SULFHYDRYL OXIDASE ACTIVITY IN SKIN HOMOGENATES*

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Among the tissues investigated for enzyme content and activity, the skin has received due attention; a comprehensive survey of research in this field has been made by Lerner (1). Certain of the enzymes found in surviving skin slices or in skin homogenates are ubiquitous, occurring in other tissues as well. Among these are hexokinase, pyruvic and succinic dehydrogenases, cytochrome oxidase, proteinases, catalase, phosphatase, and cholinesterase. Especially interesting for dermatological research, however, are enzymes which catalyze processes that occur exclusively or predominantly in the skin. Of these enzymes, tyrosinase, which is produced by the melanocytes and catalyzes the oxidation of tyrosine to melanin, has been extensively studied, and some of the details of the process of skin pigmentation have been clarified. On the other hand, very little is known about the enzymes involved in another process characteristic of the skin, namely keratinization. The present paper represents an initial effort to explore this field.

Keratinization is the transformation of cytoplasmic protein into keratin fibers, accompanied by complete disintegration of the keratinizing cell. This process consists of a number of chemical changes, most of which are complex in themselves and but little understood. These changes are thought to include (a) hydrolysis of amino-acid groups contained in the cytoplasmic proteins, thus liberating sulfur-free amino acids as well as exposing hidden —SH (sulfhydryl) groups; (b) transformation of the sulfur-containing amino acid methionine into cysteine; (c) formation of disulfide cross-linkages by oxidation of —SH groups; (d) formation of salt linkages and hydrogen bonds; and (e) glycogen synthesis followed by glycolysis, the latter supplying energy for keratin synthesis.

Undoubtedly most of these processes and subprocesses are catalyzed by enzymes. Some of these enzymes, notably those involved in glycolysis, may serve other processes in addition to keratinization. Enzymes supposedly involved in processes (a), (b), and (d) have not been directly demonstrated in the skin (or even in other tissues), their existence in the skin being assumed on the basis of indirect evidence. There is direct evidence, however, for the presence of sulfhydryl oxidase systems in various tissues other than the skin. For example, Ames and Elvehjem (2) found in cell-free homogenates of mouse kidney an enzyme system involving cytochrome *c*, cytochrome oxidase, and an enzyme defined as glutathione dehydrogenase because it oxidizes reduced glutathione, a sulfhydryl-containing compound.

Thus it appears that while sulfhydryl oxidation is an integral part of keratinization, it is not specific for that process, but can occur in other tissues for other purposes. Nevertheless, a study of it in the skin seemed to us to be of special interest because of the apparent possibility of correlating it with the varying intensity of keratinization during the hair-growth cycle.

It is known that in the epidermis keratinization proceeds more extensively during the active phase of the hair-growth cycle than during the inactive phase; and in the corium intensive keratinization goes on during the active phase, when hair keratin is rapidly produced in the hair follicles, while no keratinization occurs during the inactive phase.

In this investigation we have undertaken to study the sulfhydryl oxidase system in skin homogenates, using the Warburg technic. Reduced glutathione, cysteine, and methionine were employed as sulfhydryl sources. Homogenates of the whole skin rather than of the epidermis alone were made, so that the hair-follicle activity in the corium could be included. Colored (black) rabbits were used because sizable portions in various phases of keratinization activity are readily recognizable in this species, the skin under the hair being white in the inactive phase and highly pigmented (black) in the active phase.

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METHODS

Preparation of Homogenates. Two general types of skin homogenates were used in this study: "active" homogenates, prepared from areas of skin where hair growth was active; and "inactive" homogenates, prepared from areas where the skin was in the inactive phase. Some of the active skin homogenates were made from portions of skin which were spontaneously active in the natural course of hair-growth periodicity. Other active homogenates were prepared from portions of skin in which active hair growth had been artifically induced by epilation: all hair was manually epilated in a naturally inactive area, resulting in the onset of an intensely active phase in that area six to eight days after epilation (3). Homogenates prepared from active skin samples of these two kinds showed no significant difference in their effects on sulfhydryl oxidation.

One active and one inactive area of the skin were carefully shaved with an electric clipper, and the animal was killed by exsanguination. The selected areas were excised and fastened inside out with thumbtacks to a wooden board. The subcutaneous fat was removed with knife and forceps, and then the skin was thoroughly scarified to facilitate homogenization. The samples to be homogenized were approximately one-centimeter squares. (Kidney and liver samples were cubes about half a centimeter on an edge.) The skin was homogenized for five minutes in a Potter-Elvehjem homogenizer containing 5 ml. of M/15 potassium phosphate buffer with a pH of 7.5. The homogenate was poured off, fresh buffer was added, and the process was repeated. The two portions of homogenate were then centrifuged for five minutes at 4000 r.p.m., and the decantates were united. All vessels containing homogenates were kept in ice water as much of the time as possible.

Determination of "Tissue Concentration" in Homogenates. Dry weights and nitrogen determinations being inconvenient for our purposes, it was decided to use the protein content of the homogenates as a measure of tissue concentration. A method based on one given by Gradwohl (4) was employed as follows: 1 ml. of 5% sulfosalicylic acid was mixed with 1.5 ml. of homogenate, and after standing for five minutes, the mixture was read in a spectrophotometer at 555 mμ, using appropriate blanks. Readings were converted to concentrations by means of a table based on readings obtained with solutions containing known amounts of egg albumin. Our homogenates showed about a tenfold range of concentrations. Inactive skin homogenates had a range of 0.1 to 0.6 mg. protein per milliliter, with an average of 0.3; active homogenates had a range of 0.3 to 1.1 mg. per ml., with an average of 0.6. Presumably the higher concentrations of protein in active skin homogenates were due to the relatively greater number of cells in active skin.

Warburg Procedure. Conventional Warburg equipment was used at 37°C. As "substrates," 0.053 M neutralized solutions of reduced glutathione, L-cysteine hydrochloride, and DL-methionine were employed. "Additive" solutions included 6×10^{-4} M $CuSO_4$ in water, 1.5×10^{-5} M cytochrome *c* in buffer, and 0.02 M DL-serine in water.* Experimental flasks contained 0.3 ml. of substrate in the side arm and usually 1.0 ml. each of homogenate and any additive solution in the main chamber. Control flasks were of several types. In all experiments, there were flasks containing substrate but no homogenate, and flasks containing homogenate but no substrate. In experiments involving additives, there were also flasks containing substrate and homogenate but no additive, and flasks containing substrate and additive but no homogenate. All flasks contained sufficient buffer in the main chamber to bring the total volume of fluid up to 3.0 or 2.5 ml., depending on the experiment. The gas phase was air, and the center well of each flask contained 0.2 ml. of 10% KOH and a small strip of filter paper.

The thermal equilibration period was normally 15 minutes, at the end of which time the substrate was added to the main chamber by tilting the vessel; this was recorded as zero time for every determination. The usual time that elapsed between sacrificing the animal and zero time was two hours. Experiments comparing shorter and longer elapsed times showed only a slight decrease of catalysis or increase of inhibition after the longer time. Warburg manometers were observed until oxidation in the experimental vessels was essentially complete. This required four hours in most experiments involving glutathione, and one hour in those involving cysteine. In experiments involving methionine and serine, the manometers were observed for four hours, during which time apparently no oxidation took place.

* All organic solutes mentioned in this paragraph were purified crystalline compounds prepared by Nutritional Biochemicals Corp., Cleveland, Ohio.

RESULTS

Behavior of Homogenates Alone. In the flasks containing homogenate but no substrate, there was a small change in gas volume during the period of observation, usually several microliters, and rarely more than ten. This change was presumably due to respiration of the cell fragments in the homogenate. As is well known, respiration involves the taking up of oxygen and, ordinarily, the release of carbon dioxide. The latter gas is absorbed by the strong base in the center well of the Warburg flask, resulting in a decrease of the gas volume in the flask. However, the gas volume decreased for only 15 out of 45 homogenates; it *increased* for all the other 30. This increase is probably due to the formation of some gas which is insoluble in strong base; it could be carbon monoxide, which is known to be a product of cellular respiration under certain circumstances (5), although release of other non-acidic gases is not inconceivable.

In all graphs in this paper showing absorption of oxygen by mixtures of substrate and homogenate, the values for homogenate alone were subtracted before graphing.

Oxidation of Glutathione in the Presence of Kidney Homogenates. One experiment with mouse kidney homogenate was performed in order to compare our procedure with that of Ames and Elvehjem (2). In spite of some minor differences in technic, the results are closely comparable. Experiments with rabbit kidney homogenate gave results very similar to those obtained with mouse kidney (Fig. 1). All kidney homogenates accelerated the autoxidation of glutathione.

Oxidation of Glutathione in the Presence of Skin Homogenates. In contrast to the uniform behavior of kidney homogenates, skin homogenates showed a wide range of effects: some homogenates markedly catalyzed the autoxidation of glutathione; others had little effect, if any; and still others markedly inhibited the reaction. The effect of a homogenate can be expressed according to the following formula:

$$\% \text{ catalysis} = \frac{r_e - r_s}{r_s} \times 100\%,$$

where r_e is the (experimental) rate of oxidation in the presence of homogenate, and r_s is the (standard) rate in its absence. When r_e is less than r_s, the whole expression for catalysis becomes negative, and hence represents *inhibition.*

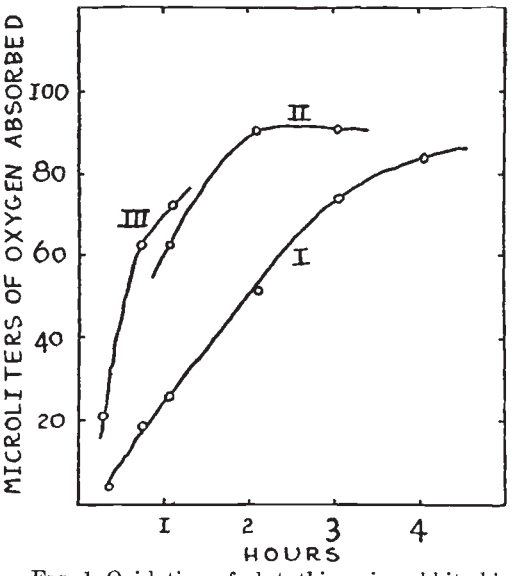

FIG. 1. Oxidation of glutathione in rabbit skin homogenate (I), in rabbit kidney homogenate (II), in mouse kidney homogenate (III).

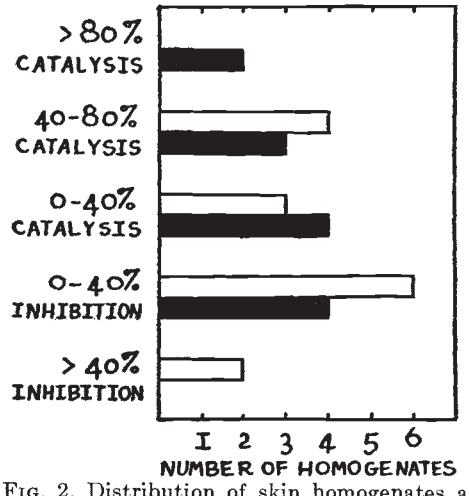

FIG. 2. Distribution of skin homogenates according to their effects on the oxidation of glutathione. Black bars represent active homogenates; white bars, inactive homogenates.

Homogenates made from inactive skins ranged from 73% inhibition to 67% catalysis, with a median of 6% inhibition and an average of 1% catalysis. Those made from active skins ranged from 26% inhibition to 163% catalysis, with a median of 35% catalysis and an average of

53% catalysis. The distribution seems to be essentially one of probability (Fig. 2). It should be noted that the oxygen absorption curves of glutathione in the presence of skin homogenates

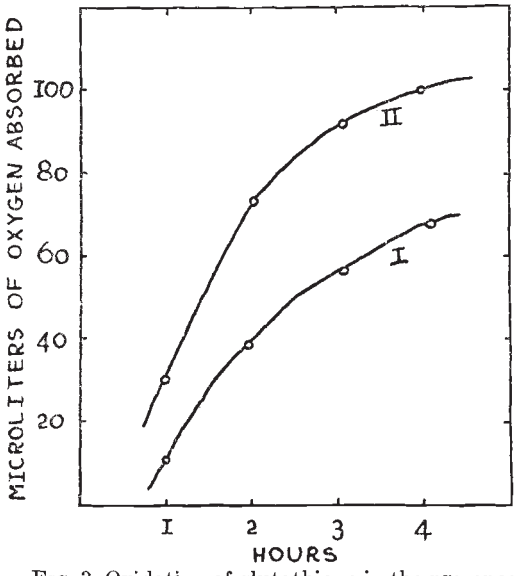

FIG. 3. Oxidation of glutathione in the presence of active rabbit skin homogenate. (Marked catalysis). I. Glutathione concentration 6.5×10^{-3} M. II. Glutathione as in I; active rabbit skin homogenate containing 0.4 mg. of protein. Total volume in each flask 2.5 ml.

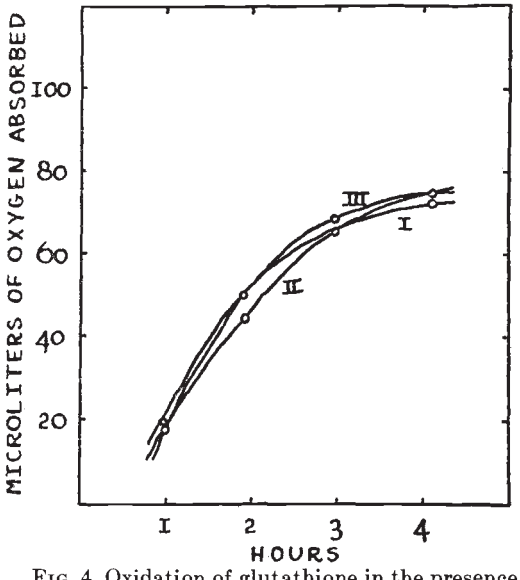

FIG. 4. Oxidation of glutathione in the presence of inactive or active rabbit skin homogenates. (Homogenates with little effect). I. Glutathione concentration 5.4×10^{-3} M. II. Glutathione as in I; inactive rabbit skin homogenate containing 0.4 mg. of protein. III. Glutathione as in I; active rabbit skin homogenate containing 0.6 mg. of protein. Total volume in each flask 3.0 ml.

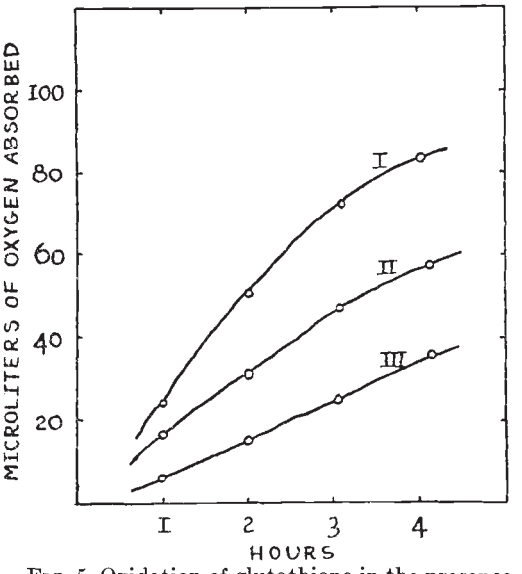

FIG. 5. Oxidation of glutathione in the presence of inactive skin homogenates. (Marked inhibition). I. Glutathione concentration 6.5×10^{-3} M. II. Glutathione as in I; 3.0 mg. dry wt. of inactive rabbit skin. III. Glutathione as in I; 3.0 mg. dry wt. of inactive mouse skin. Total volume in each flask 2.5 ml.

which are extremely catalytic, nearly ineffective, or extremely inhibitory do not differ significantly in any respect except their positions in relation to the reference curves for glutathione alone (Figs. 3, 4, and 5).

Experiments comparing the oxidation of glutathione in the presence of undiluted homogenates, and in the presence of the same homogenates diluted with buffer, showed that the influence of tissue concentration was very slight. Dilution tended to diminish the effect of the homogenate, whether catalysis or inhibition; but the difference was little even for ten-fold dilution.

Effect of Cupric Ion. It was found that cupric ion itself (in the absence of homogenate) catalyzed the oxidation of glutathione, thus confirming the work of Ames and Elvehjem (2). Cupric ion in the presence of catalytic skin homogenates produced more catalysis than the homogenates alone; but the system is not simply additive. It would seem that skin homogenates can either enhance or decrease the catalytic effect of cupric ion (Fig. 6).

Effect of Cytochrome c. The effect of skin homogenates on glutathione oxidation was not changed by the addition of 5×10^{-6} M cytochrome *c*. This is of special interest in view of

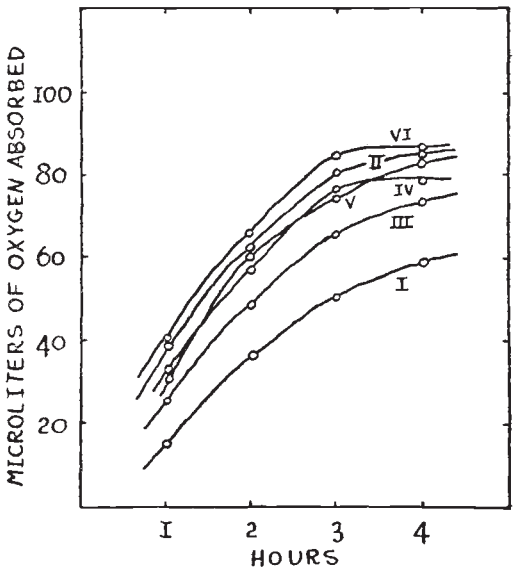

FIG. 6. Oxidation of glutathione in the presence of cupric ion and rabbit skin homogenates. I. Glutathione concentration 5.4×10^{-3} M. II. Glutathione as in I; 10^{-4} M $CuSO_4$. III. Glutathione as in I; inactive rabbit skin homogenate containing 0.4 mg. of protein. IV. Glutathione as in I; active rabbit skin homogenate containing 0.4 mg. of protein. V. Same as III, plus 10^{-4} M $CuSO_4$. Total volume in each flask 3.0 ml.

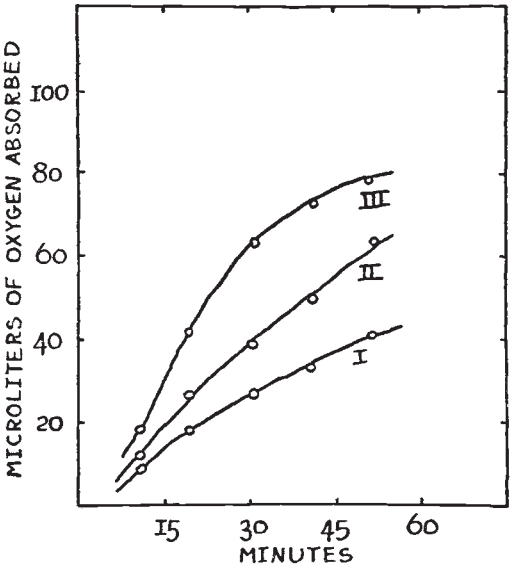

FIG. 7. Oxidation of cysteine in the presence of rabbit skin homogenates. (Marked catalysis). I. Cysteine concentration 5.4×10^{-3} M. II. Cysteine as in I; inactive rabbit skin homogenate containing 0.5 mg. of protein. III. Cysteine as in I; active rabbit skin homogenate containing 1.1 mg. of protein. Total volume in each flask 3.0 ml.

the findings of Ames and Elvehjem (2) that such a concentration of added cytochrome *c* greatly increases the rate of glutathione oxidation catalyzed by kidney homogenates.

Oxidation of Cysteine in the Presence of Kidney or Liver Homogenates. The oxidation of cysteine was completely unaffected by the presence of rabbit kidney or liver homogenates. This is to be contrasted with the oxidation of glutathione, which is greatly catalyzed by the addition of kidney homogenates.

Oxidation of Cysteine in the Presence of Skin Homogenates. The effect of skin homogenates on the oxidation of cysteine was found to be essentially the same as on the oxidation of glutathione. In one experiment, both inactive and active skin homogenates were moderately inhibitory; in another, both homogenates were greatly catalytic. Probably the effects of skin homogenates on the oxidation of cysteine are as widely variable as on that of glutathione. The only difference between the curves showing oxygen consumption by cysteine and by glutathione is in the time scale, the former curve indicating about four times as rapid oxidation as the latter (Fig. 7).

Behavior of Methionine and Serine in the Presence of Kidney, Liver, or Skin Homogenates. If a mixture of methionine and serine were to be converted to cysteine in the presence of tissue homogenate, any cysteine thus formed would be rapidly oxidized, and the volume of oxygen taken up would be a measure of the extent of conversion. It was found, however, that solutions 5.3×10^{-3} M in both methionine and serine absorbed essentially no oxygen in the presence of kidney, liver, or skin homogenates, indicating that no cysteine was formed under the conditions of these experiments.

DISCUSSION

Our findings seem to indicate that rabbit skin homogenates contain both catalyst(s) and inhibitor(s) for sulfhydryl oxidation, the resultant effect—whether acceleration or inhibition—depending on the relative concentrations of these substances. It appears that homogenates prepared from active skin (in which keratinization proceeds at a rapid rate) tend to contain more catalyst and/or less inhibitor as compared with homogenates from inactive skin, which tend to contain less catalyst and/or more inhibitor. (It may be of interest in this connection that Griesemer and Gould (6) recently reported prelim-

inary experiments which showed more activity of succinic dehydrogenase and cytochrome oxidase in homogenates of rat epidermis during the growth phase of the hair cycle than during the dormant phase.)

Are the inhibitors in the skin homogenates artifacts which do not operate *in vivo*, or do they have specific biological functions in regulating sulfhydryl oxidation in the process of keratinization? In trying to answer this question, it may be useful to compare the effects of skin homogenates on sulfhydryl oxidation first with *the effect of kidney homogenates on the oxidation of glutathione*, and then with *the effect of skin homogenates on the oxidation of tyrosine to melanin.*

The effect of kidney homogenates on the oxidation of glutathione and the effects of skin homogenates on sulfhydryl oxidation have certain definite differences, as our results indicate. It seems to us that at least some of these differences could be reasonably explained as differences in the systems of inhibitors existing in the tissues concerned. Apparently inhibitors do not play an important role in the glutathione oxidase system of the kidney or liver: the inhibitors, if any, in these homogenates do not materially vary in amount or effectiveness, and hence cannot represent major regulatory factors. Possibly the glutathione oxidase system in kidney or liver homogenates may not be a reflection of any biological function *in vivo*. On the other hand, the peculiarities of the corresponding enzyme system in skin homogenates may be regarded as supporting the view that it serves a specific function not shared by other tissues.

As was mentioned earlier, Ames and Elvehjem (2) found that cytochrome *c* added to kidney homogenates greatly accelerates the oxidation of glutathione. From this observation these authors concluded that cytochrome *c* (and cytochrome oxidase) are essential elements of that enzyme system. We have found that added cytochrome *c* has no effect on the oxidation of glutathione in the presence of skin homogenates. This may mean either that cytochrome *c* occurs naturally in skin homogenates in amounts sufficient for whatever role it may play in catalyzing the oxidation of glutathione in the presence of skin homogenates, or else that it plays no such role at all, and the oxidation proceeds along some other pathway. Be that as it may, here is another feature of sulfhydryl oxidation apparently specific for the skin.

Even more striking is the difference between skin homogenates and kidney (or liver) homogenates in their effects on cysteine autoxidation: the former apparently have the same variable effects on the oxidation of cysteine as on that of glutathione; while the latter, which uniformly catalyze the oxidation of glutathione, have no effect whatever on that of cysteine. This difference, if confirmed, again strongly underlines the different character of the sulfhydryl oxidase system existing in the skin.

For the time being, the identity of the sulfhydryl oxidase inhibitors present in skin homogenates remains unknown. Ames and Elvehjem (2) investigated the effect of certain known antioxidants added to kidney homogenates on glutathione oxidation—not because they suspected the presence of such inhibitors in their homogenates, but because they expected to learn in this way something about the nature of the enzyme. They found that when small amounts of cyanide or diethyldithiocarbamate (substances known to bind copper) were added to the kidney homogenate—glutathione system, oxidation was completely inhibited. On this basis they concluded that the glutathione oxidase system in their homogenates involves in all probability a copper-protein enzyme. They also found that added iodoacetamide, presumed to react with the —SH group of the glutathione and thereby reduce the concentration of the substrate, strongly inhibited the oxidation. Of course, none of these antioxidants are normally present in tissue homogenates. However, certain biological substances, such as ascorbic acid, are known antioxidants. Much further work will be necessary to learn the identity of the sulfhydryl oxidase inhibitors in skin homogenates.

The effect of skin homogenates on the oxidation of tyrosine to melanin is much better understood than the effect of skin homogenates on sulfhydryl oxidation. The enzyme tyrosinase normally exists in an inhibited state in the epidermis, but its presence there has been histochemically demonstrated after releasing it from its inhibited state by repeated ultraviolet irradiation. Active mammalian tyrosinase has been extracted from melanomas (7) but has never been demonstrated in skin homogenates; that this may be due to the presence of inhibitory substances in skin homogenates is evident from the fact that the action of purified tyrosinase (prepared from mushroom

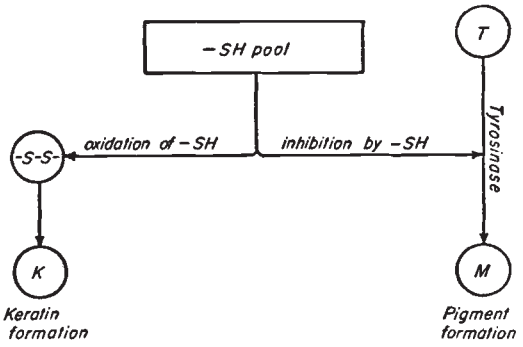

FIG. 8. Diagram illustrating dependence of rate of pigment formation on the rate of keratin formation, in the hair. (See text.) T stands for Tyrosine; M, for Melanin; K, for Keratin; —SH, for sulfhydryl groups; —S—S—, for bisulfide linkages.

or potato) on tyrosine *in vitro* is completely inhibited by the addition of skin homogenate. It has been found that tyrosine-tyrosinase systems can be inhibited by a number of factors acting through several mechanisms, such as direct enzyme poisons, competitive inhibitors, reducing agents, and inhibitors with unknown modes of action. It has been suggested on the basis of several lines of evidence that the actual inhibition in the epidermis may be a function of sulfhydryl compounds normally present in the epidermis, probably acting by their suppressive effect on the copper moiety of tyrosinase. A fruitful hypothesis has been advanced by Rothman and his associates concerning the regulatory role of these inhibitory sulfhydryl compounds in the physiological and pathological formation of pigment in the skin (8, 9).

The fact remains, however, that an inactive skin may be catalytic, and an active skin may be inhibitory, which indicates that factors other than those connected with keratinization may also be important in determining the over-all effect on sulfhydryl oxidation.

Thus it appears that both major skin-specific enzymes—tyrosinase and sulfhydryl oxidase—are regulated by biological inhibitors. Sulfhydryl compounds may function as connecting links between pigmentation and keratinization in the hair. When pigmented hair grows, pigment must be formed and deposited into the hair in direct proportion to the rate of keratin formation in the hair. Conceivably, the two rates are proportionalized by the following mechanism: (a) as hair growth proceeds, sulfhydryl compounds are oxidized and built into keratin; (b) removal of these compounds represents removal of tyrosinase inhibitors and allows pigment formation in proportion to the rate of removal (Fig. 8).

As was mentioned earlier, oxidation of sulfhydryl groups is but one of a number of chemical changes that make up the keratinization process. The rate and timely sequence of all these changes must be closely harmonized when normal hair growth is maintained. This must involve a whole series of interdependent regulatory mechanisms, and the action of sulfhydryl oxidase inhibitors may very likely represent one of these mechanisms.

SUMMARY

1. The effects of rabbit skin homogenates on the autoxidation of glutathione and cysteine were studied. It was found that the effects varied from marked inhibition to marked acceleration.

2. Homogenates prepared from skin in the inactive phase of the hair-growth cycle showed a tendency towards inhibition of sulfhydryl oxidation, as compared with homogenates prepared from skin in the active hair-growth phase, which showed a tendency towards acceleration. It is suggested, on this basis, that the sulfhydryl oxidase activity in skin homogenates is connected with the keratinization process. However, the parallelism of the two processes is far from being strictly consistent, indicating that some role in the sulfhydryl oxidase activity of skin homogenates is played by factors other than those involved in keratinization.

3. The possible significance of sulfhydryl oxidase inhibitors in the regulation of keratinization is discussed, and a theory is proposed concerning the close relationship between the rate of pigment production and the rate of keratinization during the growth of colored hair.

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DISCUSSION

DR. PETER FLESCH (Philadelphia, Pa.): I would like to ask Dr. Rony, whether he has used any boiled homogenates since organic substances will also catalyze the oxidation of glutathione.

DR. HUGO RONY (in closing): Ames and Elvehjem reported that boiling destroyed the activity of the sulfhydryl enzyme system in guinea pig kidney homogenates. They found that 0.002 M cyanide, too, completely inhibited that enzyme system, and suggested that this indicates that the enzyme contains a copper moiety. We found that 0.002 M cyanide inhibited only partially the catalysis of catalytic rabbit skin homogenates, and that it completely canceled out the inhibition of inhibitory skin homogenates. This suggests that the catalysts in kidney homogenates and in (catalytic) skin homogenates are different substances, and that the inhibitor in (inhibitory) skin homogenates acts by catalyzing a side reaction which interferes with the main reaction.